Monophenolase Activity of Polyphenol Oxidase from Haas Avocado

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Avocado polyphenol oxidase (PPO) has been isolated and partially purified by using Triton X-114, reported here for the first time. The enzyme showed both monophenolase and diphenolase activities. The monophenolase activity of avocado PPO was characterized using 4-hydroxyanisole (4HA) as substrate with 3-methyl-2-benzothiazolinone hydrazone as coupled nucleophile. This continuous spectrophotometric method was reliable, with high sensitivity and precision. 4HA was a very good substrate for avocado PPO, showing a higher k_{cat} than that for L-dopa at the optimum pH. Monophenolase activity of PPO showed a lag period (τ) prior to the attainment of the steady state rate (V_{ss}). The optimum pH was 5, the same for the monophenolase and diphenolase activities when the *p*-hydroxyphenylpropionic acid/3,4-dihydroxyphenylpropionic acid pair was assayed, as well as when 4HA was assayed. A reaction mechanism for explaining the kinetic behavior of the monophenolase activity of avocado PPO has been proposed and characterized.

Keywords: *Avocado; enzyme kinetics; 4-hydroxyanisole; MBTH; monophenolase; polyphenol oxidase*

INTRODUCTION

Polyphenol oxidase (PPO) is a copper enzyme which in the presence of oxygen catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red, or black pigments (Mason, 1955; Prota, 1988). PPO has been the subject of several reviews (Vámos-Vigyázó, 1981; Robb, 1984; Mayer, 1987; Nicolas et al, 1994; Sánchez-Ferrer et al., 1995) because enzymatic browning in fruits and vegetables provokes unpleasant sensory qualities and losses in nutrient quality. The prevention of this reaction has always been a challenge to food scientists (Matheis, 1987).

For most PPOs, including avocado PPO, diphenolase activity has been widely characterized (Benjamin and Montgomery, 1973; Khan, 1976a,b; Halim and Montgomery, 1978; Lelyveld et al., 1984; Janovitz-Klapp et al., 1990; Heimdal et al., 1994). However, there are few studies on monophenolase activity (Rodríguez-López et al., 1992, 1994; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b) and only one where the monophenolase activity of avocado PPO is described (Kahn and Pomerantz, 1980). This scarce information about the monophenolase activity of PPO stems from the lability of the enzyme during the purification process (Matheis, 1987). This phenomenon is well-known in other plant PPOs (Mayer and Harel, 1979) and results from changes in the structure of the protein during purification (Walter and Purcell, 1980).

Avocado PPO was located as both soluble and membrane-bound forms (Mayer and Harel, 1979; Lelyveld et al., 1984). The monophenolase activity of avocado PPO was detected from an enzyme extracted by using an acetone powder (Kahn and Pomerantz, 1980). This extraction protocol yielded an extract with low specific activity since the highest value was 0.076 μM min^{-1} (mg of avocado protein)$^{-1}$, when *p*-cresol was assayed. The monophenolase activity was measured by using the radiometric method of Pomerantz (1964) and the spectrophotometric method that measures the change in absorbance at 475 nm (Mason, 1948). Both assay methods were discontinuous and required very long assay times [see Figure 1 from Kahn and Pomerantz (1980)]. The limitations of these extraction and detection methods make it difficult to understand the reaction mechanism of avocado PPO.

The aim of this paper is the detection and kinetic characterization of the monophenolase activity of avocado PPO, after its extraction and partial purification by using a method with Triton X-114 (TX-114) previously applied to other PPOs (Espín et al., 1995a,b, 1996a,b). The monophenolase activity was assayed with 3-methyl-2-benzothiazolinone (MBTH) as a coupled chromogenic nucleophile (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996a,b) on *p*-hydroxyphenylpropionic acid (PHPPA) and 4-hydroxyanisole (4HA) as new substrates for avocado PPO. From the kinetic characterization of the monophenolase activity of this enzyme, the applicability to avocado PPO of the same reaction mechanism previously proposed for PPO from other sources (Cabanes et al., 1987; Rodríguez-López et al., 1992; Ros, et al., 1994; Espín et al., 1995a,b, 1996a,b) was tested.

MATERIALS AND METHODS

Reagents. Avocados from the variety Haas imported from Israel at commercial maturity and stored at 13 °C were used as enzyme source.

4HA was purchased from Aldrich (Madrid, Spain), DHPPA, PHPPA, and MBTH were from Sigma (Madrid, Spain), and all other reagents were of analytical grade.

Triton X-114 was obtained from Fluka (Madrid, Spain) and condensed three times prior to use as described by Bordier (1981) but using sodium phosphate buffer (PB) pH 7.3, containing 20 mM EDTA. The detergent phase of the third condensation had a concentration of 22% TX-114 (w/v).

Preparation of PPO. Avocado PPO was extracted by using the method with TX-114 which was previously applied to extract apple (Espín et al., 1995a,b) and pear (Espín et al., 1996a,b) PPO.

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A 150 g sample was homogenized with 300 mL of cold buffered 0.1 M sodium phosphate, pH 7.3, 20 mM EDTA, and 4% (w/v) TX-114 for 2 min. The homogenate was kept at 4 °C for 60 min before being centrifuged at 120000*g* for 30 min at 4 °C. The supernatant was collected and used as a crude enzyme extract. It was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 6% (w/v) at 4 °C and then warming to 35 °C for 15 min. The solution became turbid due to the formation, aggregation, and precipitation of large micelles of detergent which contained hydrophobic proteins and phenolic compounds. This solution was centrifuged at 8000*g* for 15 min at 25 °C. The detergent-rich phase was discarded, and the supernatant was subjected to an additional phase-partitioning step with 6% (w/v) TX-114. The protocol was repeated twice more in order to remove the remaining phenols. The supernatant brought to 30% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 15 min, the solution was centrifuged at 80000*g* for 30 min at 4 °C and the pellet discarded. Additional $(NH_4)_2SO_4$ was added to the clear supernatant to give 80% saturation, and the resultant mixture stirred for 30 min at 4 °C. The solution was centrifuged at 100000*g* for 30 min and the precipitate dissolved in a minimal volume of deionized water. The salt content was removed by a desalting column of Sephadex G-25. The enzyme was stored at −30 °C without loss of the original activity after 1 month and no discoloration. This simple and fast protocol gave a 8-fold purification of the enzyme extract, which preserved its monophenolase activity.

Other Methods. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Enzymatic Assays. The use of MBTH as a nucleophilic agent on some *o*-quinones generated by PPO was previously described for the measurement of diphenolase (Winder and Harris, 1991) and monophenolase (Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996a,b) activities of PPO. The solubility of the chromophoric MBTH−quinone adducts required the use of 2% DMF in the assay medium.

The diphenolase activity was assayed spectrophotometrically at 500 nm using as substrate 3,4-dihydroxyphenylpropionic acid (DHPPA) with MBTH as coupled nucleophile.

The monophenolase activity was also determined spectrophotometrically at 500 nm with PHPPA and at 464 with 4HA using MBTH as coupled nucleophile.

One unit of enzyme was taken as the amount that produced 1 μmol of the adduct/min. Experiments were performed at 25 °C in triplicate, and the corresponding mean values were plotted.

The spectrophotometric assays were carried out with a Perkin-Elmer Lambda-2 spectrophotometer on-line interfaced with a compatible-PC computer for further data analysis. All the assays were carried out at 25 °C with a Haake D1G circulating water bath equipped with a heater/cooler and controlled by a Cole-Parmer digital thermometer with a precision of ±0.1 °C.

Data Analysis. Kinetic data analysis was carried out by using linear and non linear regression fittings (Marquardt, 1963), using the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994).

Determination of the enzyme concentration was carried out by lowering the PPO concentration to a level where V_{ss} was 10-fold higher than the rate of nonenzymatic oxidation of the 4HA substrate (blank rate). The cuvette contained saturating concentration of monophenolic substrate and enough MBTH concentration to trap all the generated *o*-quinone. Ten blank cuvettes were assayed for the determination of the limit of detection (LOD) and the limit of quantitation (LOQ) of the method (ACS, 1980). The precision of the method was also evaluated from 10 activity assays at each one of the three enzyme concentrations used (Espín et al., 1995a,b, 1996a,b).

RESULTS AND DISCUSSION

Avocado PPO was extracted and partially purified by using two sequential phase partitionings with TX-114 for the first time (Espín et al., 1995a,b, 1996a,b). The enzyme had both diphenolase (Figure 1a) and monophenolase activities (Figure 1b). The latter was characterized by a lag period (τ) prior to the attainment to the steady state rate (V_{ss}) (Espín et al., 1995a,b, 1996a,b). This extraction method permits one to obtain an enzyme with a high specific activity (442 μM min^{-1} (mg of avocado protein)$^{-1}$, when 4HA was assayed). Drastic extraction methods provoke the loss of the monophenolase activity (Mayer and Harel, 1979; Walter and Purcell, 1980; Matheis, 1987). The catalytic power (V_{max}/K_m) for the diphenolase activity is much higher than that for the monophenolase activity of PPO (Rodríguez-López et al., 1992; Ros et al., 1994). Thus, losses in the PPO activity lead to a very low catalytic power for the monophenolase activity.

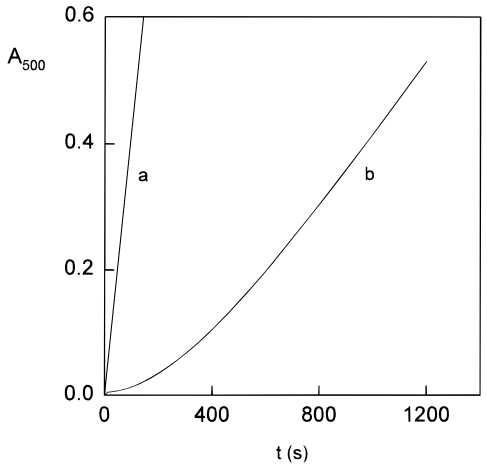


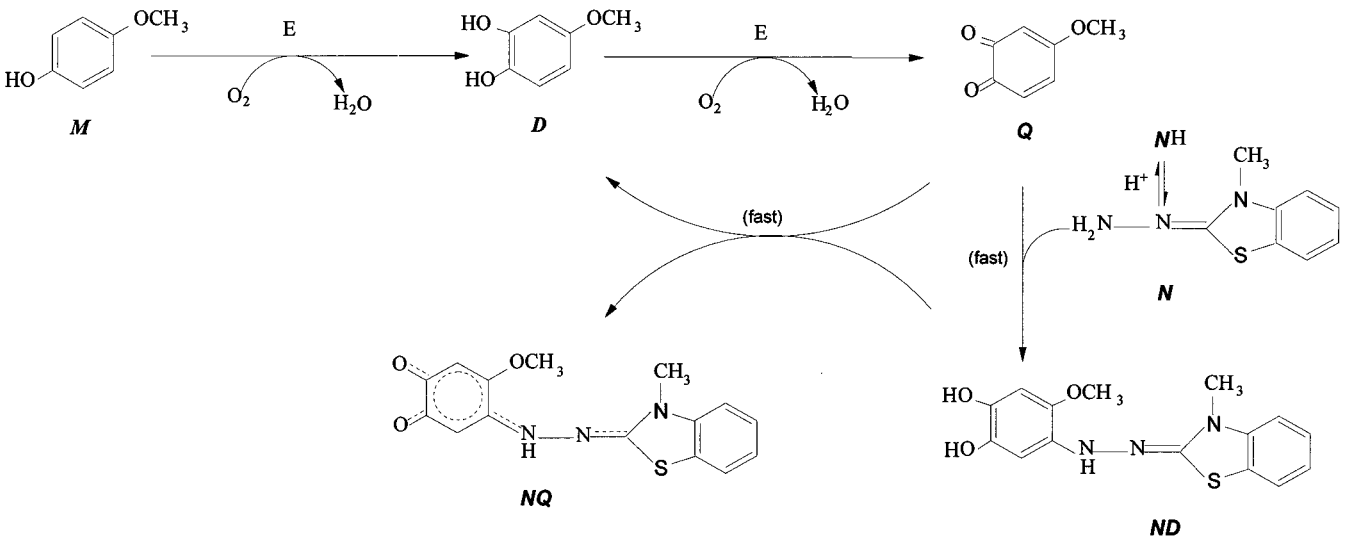
Figure 1. Enzymatic activities of soluble avocado PPO: (a) Diphenolase activity. The reaction medium included 2 μg/mL avocado protein, 6 mM DHPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate buffer (AB) (pH 4.5). (b) Monophenolase activity. The reaction medium contained 12 μg/mL avocado protein and 3 mM PHPPA in 50 mM AB (pH 4.5).

Determination of the Enzyme Activity. In this work we used an assay method previously described (Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996a,b) which is based on the coupling reaction between MBTH and the enzyme generated *o*-quinones, yielding a chromophoric MBTH−quinone adduct with high stability and molar absorptivity following the stoichiometry

2 *o*-quinone + 1 MBTH →
1 MBTH−*o*-quinone adduct + 1 *o*-diphenol

To study the characteristics of the MBTH−quinone adduct, the oxidation of 4HA by avocado PPO was carried out in the presence of MBTH. The pigment formed was reddish in color and had an absorbance maximum of 492 nm. The pH affected the solubility and stability of the adduct. The solubilization of this compound was achieved by adding 2% (v/v) DMF to the reaction medium (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a, 1996a). Under these conditions, the adduct formed by the addition of MBTH to the *o*-quinone was soluble at every pH studied (from 3.5 to 7). At values of pH lower than 5, the adduct was soluble and stable, whereas at higher values, the adduct was unstable but showed an isosbestic point at 464 nm (results not shown) (Espín et al., 1995a, 1996a). MBTH is a potent nucleophile through its amino group, which is in different degrees of protonation−deprotonation, depending on the pH. The ionization constant

Scheme 1. Sequence of Reactions in the Oxidation of 4HA by Avocado PPO in the Presence of MBTH[a]



[a] *M*, monophenol; *D*, *o*-diphenol; ***Q***, *o*-quinone; *N*, nucleophile; *ND*, nucleophile−*o*-diphenol colorless adduct; *NQ*, nucleophile−*o*-quinone chromophoric adduct (MBTH−*o*-quinone adduct).

for this group has been determined by both potentiometric and spectrophotometric methods ($pK_a = 5.8 \pm 0.4$). The saturating MBTH concentration was determined by measuring the initial rate of change in absorbance, using different amounts of MBTH (results not shown) (Rodríguez-López et al., 1994; Espín et al., 1995a, 1996a).

In this work, we used 4HA as the monophenolic substrate to characterize the monophenolase activity of avocado PPO (Scheme 1). This monophenol is a very good substrate for avocado PPO because the effect of the methoxyl group on the hydroxyl group of the aromatic ring facilitates the nucleophilic attack of the oxygen to the copper of the active site of the enzyme. The enzyme showed even more activity on the monophenol 4HA than on the *o*-diphenol L-dopa ($V_{max}^{4HA} = 5.3$ μM/min; $V_{max}^{DOPA} = 3.2$ μM/min) in the same assay conditions.

The continuous spectrophotometric method used in this work has several advantages over the radiometric method which measures the tritium released as water from L-[3,5-^{3}H]tyrosine. We demonstrated in a previous work (Rodríguez-López et al., 1992) that the measure of the dopachrome formation is equal to the tyrosine consumption and to the tritiated water formation in the steady state, but if there is dopachrome evolution to indole, which is oxidized by the *o*-quinone with consequent breaking of the steady state (Mason, 1955; Prota, 1988) in very long kinetic assays, this can provoke a prolonged curvature in the dependency of tritiated water formation vs time (Khan and Pomerantz, 1980). Our assay with MBTH detects the monophenolase activity in much shorter assay times in which a stable chromophoric product is measured.

Effect of pH. Monophenolase activity toward 4HA increased as the pH was increased from pH 3.5 and showed a maximum at pH 5 (Figure 2). The pH affected not only the enzyme activity but also the lag period. Both plots, V_{ss} vs pH and τ vs pH, showed the same profiles (Figure 2). These results were not similar to those described for some plant PPOs (Cabanes et al., 1987; Sánchez-Ferrer et al., 1988; Valero et al., 1988) but were in agreement with those obtained for other plant PPO (Ros et al., 1994; Espín et al., 1995b, 1996b). The profile of τ vs pH was explained because the E_{met} form of the enzyme (inactive on monophenols) had more affinity with monophenols and therefore τ increased. However, when the pH was either lower or higher than the optimum, the affinity of the E_{met} form of the enzyme for monophenols was lower and τ decreased. When the PHPPA/DHPPA pair was assayed, the same optimum pH of 5 was obtained for both substrates (data not shown). The kinetic constants were $K_m^M = 0.3$ mM, $V_{max}^M = 0.6$ μM/min, $K_m^D = 3.4$ mM, and $V_{max}^D = 30$ μM/min.

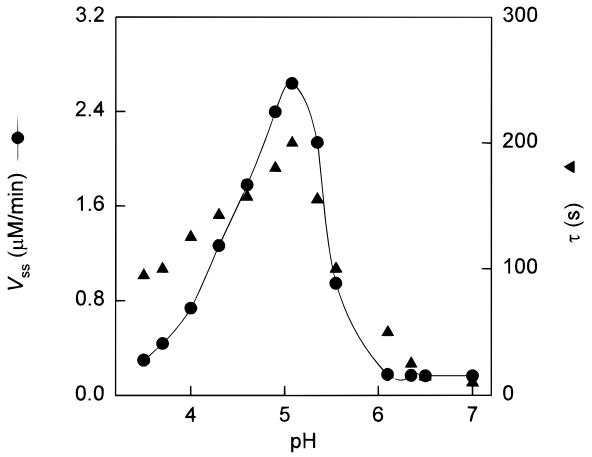


Figure 2. Effect of pH on monophenolase activity (●) and on its lag period (▲). The reaction medium included 12 μg/mL avocado protein, 3 mM MBTH, 2% DMF, and 1 mM 4HA in 50 mM AB (pH 3.6−5.6) and sodium phosphate buffer (PB) (pH 5.8−7.0).

Effect of the Enzyme Concentration. An increase of the enzyme concentration produced a linear increase in V_{ss} as well as a hyperbolic shortening in the lag period (Figure 3). This behavior has been widely described from several PPO sources (Cabanes et al., 1987; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b). The data obtained can be explained by Scheme 2 (Rodríguez-López et al., 1992). Increasing PPO concentrations produce a proportional increase of the E_{oxy} form in the native state, which means that there is more enzymatic activity, the level of *o*-diphenol in the steady state is reached more quickly, and so, τ diminishes (Figure 3).

Effect of Monophenol Concentration. An increase of monophenol concentration produced an in-

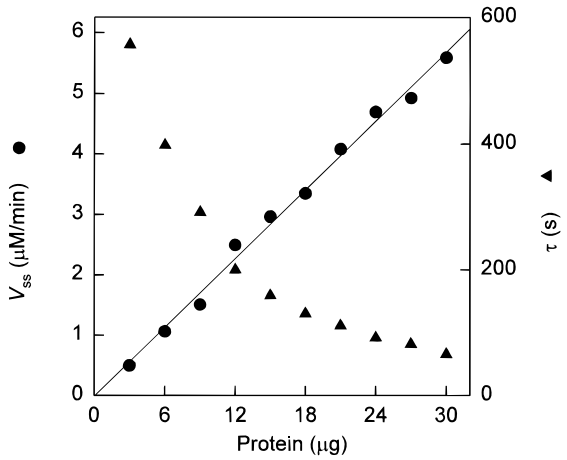
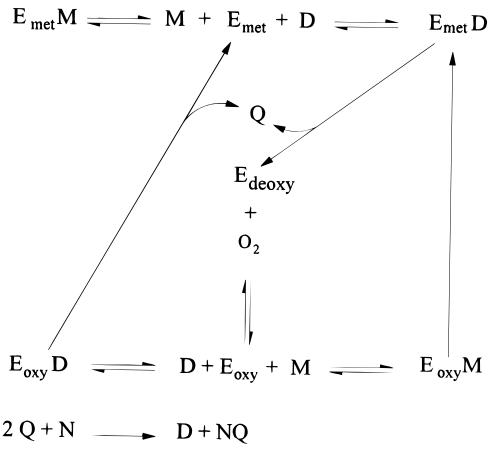


Figure 3. Effect of enzyme concentration on monophenolase activity of avocado PPO (●) and on its lag period (▲). The reaction medium included 1 mM 4HA, and 2% DMF, 3 mM MBTH in 50 mM AB (pH 5) with different avocado protein concentrations (3−30 μg/mL).

Scheme 2. Kinetic Reaction Mechanism for the Monophenolase and Diphenolase Activities of Avocado PPO Coupled to Nonenzymatic Reactions from *o*-Quinone[a]



[a] E_{met}, mettyrosinase or oxidized form of tyrosinase with $Cu^{2+}-Cu^{2+}$ in the active site; E_{deoxy}, reduced form of tyrosinase with $Cu^{+}-Cu^{+}$ in the active site; E_{oxy}, oxytirosinase ($E_{deoxy}O_2$ or $E_{met}O_2^{2-}$); M, monophenol; D, diphenol; Q, *o*-quinone; N, nucleophile (MBTH); NQ, MBTH−*o*-quinone adduct.

crease in both the steady state rate and lag period (Figure 4), similar to that described for other PPOs (Lavollaly et al., 1975; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b). This behavior can be explained by Scheme 2 (Rodríguez-López et al., 1992). The E_{met} form of the enzyme is saturated when the monophenol concentration is raised, and there is more enzyme in the dead-end complex $E_{met}M$ requiring more time to reach the steady state. Moreover, the dimensionless parameter R, which is the ratio $[D]_{ss}/[M]$, must be achieved (Rodríguez-López et al., 1992; Ros et al., 1994). Therefore, an increase in the monophenol concentration produces an increase in the *o*-diphenol concentration and more turnovers must achieve the E_{oxy} form of the enzyme (which is always the same) to accumulate the necessary *o*-diphenol.

Effect of *o*-Diphenol Addition. The addition of different *o*-diphenol concentrations in the monophenolase activity diminished the lag period (Figure 5). The data obtained can be explained by Scheme 2 (Rodríguez-López et al., 1992). The R value $[D]_{ss}/[M]$ is constant (Rodríguez-López et al., 1992; Ros et al., 1994). There-

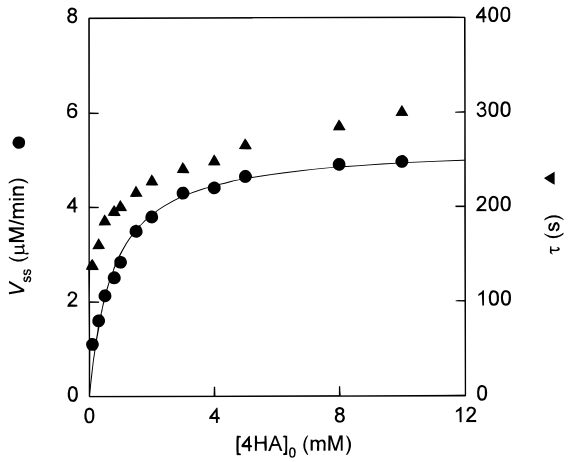


Figure 4. Effect of 4HA concentration on monophenolase activity of avocado PPO (●) and on its lag period (▲). The reaction medium included 12 μg/mL of avocado protein, 3 mM MBTH, and 2% DMF in 50 mM AB (pH 5) with different 4HA concentrations (0.1−10 mM).

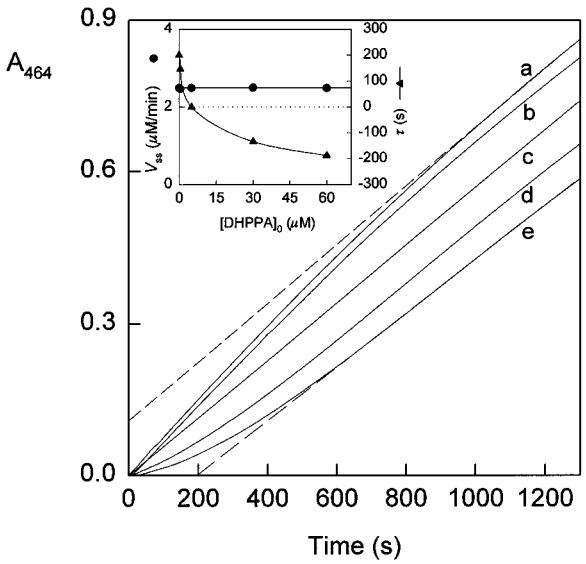


Figure 5. Influence of the initial *o*-diphenol concentration on the lag period of monophenolase activity. In the standard reaction with 12 μg/mL avocado protein, 1 mM 4HA, 3 mM MBTH, 2% DMF, and 50 mM AB (pH 5), the concentration of DHPPA was (a) 0, (b) 0.5, (c) 5, (d) 30, and (e) 60 μM. (Inset) Effect of catalytic amounts of $[DHPPA]_0$ on the steady state rate of monophenolase activity (●) and on its lag period (▲). The conditions were the same as detailed above.

fore, a raise in the initial *o*-diphenol concentration ($[D]_0$) shortened the time required for the steady state rate level *o*-diphenol ($[D]_{ss}$) to be reached. When the $[D]_0$ is higher than $[D]_{ss}$, the system must first consume the excess of *o*-diphenol and then gradually consume the monophenol and *o*-diphenol before the steady state rate is finally reached. There was a burst in the activity under these conditions. Depending on the assay conditions, a $+\tau$ of $-\tau$ appeared (Figure 5, inset) which corresponded with an over-steady-state or sub-steady-state rate level of $[D]_0$, respectively.

Determination of the Enzyme Concentration. Consideration of the above factors allows for the determination of the optimal monophenolase assay conditions on 4HA. Thus, a LOD of 0.11 pkat/mL and a LOQ of 0.13 pkat/mL were obtained (Figure 6). Furthermore, from 10 assays at 1.1, 5.5, and 11 pkat/mL enzyme

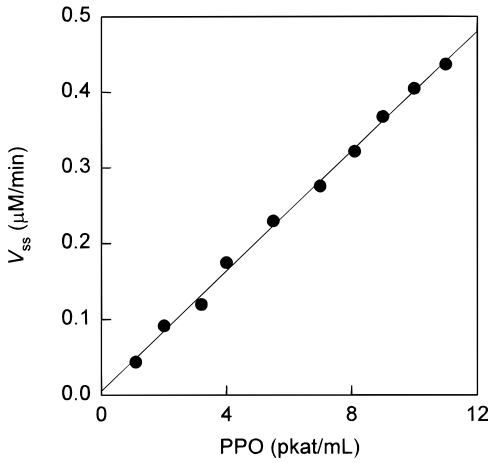


Figure 6. Effect of enzyme concentration on monophenolase activity of avocado PPO using 4HA with MBTH. Determination of LOD and LOQ (see text for details). The reaction medium contained 3 mM 4HA, 3 mM MBTH, 12 μM DHPPA, 2% DMF, and 50 mM AB (pH 5) and different concentrations of avocado PPO (1−10 pkat/mL).

concentrations, CVs of 4.6, 3.9, and 2%, respectively, were obtained for the corresponding V_{ss} data values (Figure 6).

CONCLUSIONS

Avocado PPO extracted with TX-114 showed high activity on monophenols. 4HA was a very good substrate, and its *o*-quinone formed a soluble, stable, and sensitive chromophoric adduct with MBTH (Scheme 1). The continuous spectrophotometric method used was reliable, sensitive, and precise (Figure 6). The enzyme fulfilled all the kinetic tests (Figures 2−5) of the reaction mechanism previously proposed for PPOs from other sources. Therefore, this mechanism was also applicable to the monophenolase activity of avocado PPO, which was systematically characterized for the first time. (Scheme 2).

ABBREVIATIONS USED

AB, sodium acetate buffer; CV, coefficient of variation; $[D]_0$, initial *o*-diphenol concentration; $[D]_{ss}$, *o*-diphenol concentration in the steady state; DHPPA, 3,4-dihydroxyphenylpropionic acid; DMF, *N*,*N*′-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; 4HA, 4-hydroxyanisole; K_m^D, Michaelis constant of PPO toward *o*-diphenols; K_m^M, apparent Michaelis constant of PPO toward monophenols; LOD, limit of detection; LOQ, limit of quantitation; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PB, sodium phosphate buffer; PHPPA, *p*-hydroxyphenylpropionic acid; PPO, polyphenol oxidase (EC 1.14.18.1); TX-114, Triton X-114; V_{max}^D, maximum steady state rate of PPO toward *o*-diphenol; V_{max}^M, maximum steady state rate of PPO toward monophenol; V_{ss}, steady state rate.

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